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Review Article

Applications of Flow Cytofluorometry to Red Blood Cell Immunology

George Garratty* and Patricia A. Arndt
Southern California Region, American Red Cross Blood Services, Los Angeles, California

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Agglutination and/or hemolysis of red blood cells (RBCs) by blood group antibodies have been used as indicators of antigen-antibody reactions by immunohematologists, since Landsteiner discovered the ABO blood groups in 1901. Agglutination utilizing a secondary antibody (e.g., sensitization of RBCs with nonagglutinating primary blood group antibody, followed by agglutination by a secondary antibody, anti-human globulin (AHG)) has been used since the description of the antiglobulin test (AGT) in 1945. Agglutinating antibodies are usually IgM and are often ''naturally occurring'' (no known immunogen), (e.g., anti-A, -B, -M, -N, -P_1, -Le^a, -Le^b). Nonagglutinating antibodies are usually IgG (e.g., -Rh, -K, -Fy^a, -Jk^a) and the result of a known immunogen (''immune''), usually associated with transfusion or pregnancy. Sometimes, naturally occurring agglutinating antibodies (e.g., anti-A, -B) have an IgG component, and less commonly IgM antibodies can be nonagglutinating antibodies.

When we first thought about the possibility of applying flow cytofluorometry (FC) to RBC immunology, we were pessimistic, as it seemed to us that even small agglutinates would interfere with a technique measuring interactions at a single-cell level. Agglutination could be caused by the primary antibody or the secondary antibody (e.g., AHG conjugates). We were stimulated to start applying FC after reading a successful application by van der Meulen et al. (1). They applied FC to measure the relative amount of IgG on RBCs of patients who had developed positive direct antiglobulin tests (DATs).

Our first approach to overcome the agglutination problem was simply to vortex the agglutinated RBCs, which appeared to be successful, for our early applications (2). Some years later we found that the success was not so much due to the vortexing but rather mixing of RBCs with a small bore (0.5-mm) pipette, that was performed before the vortexing and flow cytometric analysis (3). This simple approach is adequate for disrupting most of the friable agglutination caused by AHG and can be used for investigations where precise quantitation is not important. It is not an adequate approach when powerful agglutinating antibodies (e.g., ABO) are involved or where small agglutinates (e.g., doublets) will interfere with the accuracy of results (e.g., quantitating minor cell populations). To overcome strong agglutination by primary antibody, a nonagglutinating (this can sometimes be achieved by dilution) primary antibody should be used; if this is not possible, RBCs can be treated with chemicals such as glutaraldehyde, formaldehyde (with and without sodium dodecyl sulfate), and dimethylsuberimidate (4–6). Such chemicals allow uptake of antibody but greatly diminish crosslinking and agglutination. Problems that can occur with chemical fixation of RBCs include in vitro hemolysis, a decrease in antigen strength, nonspecific antibody/protein uptake, and an increase in autofluorescence (6,7). To overcome the much more common problem of agglutination associated with the secondary antibody (e.g., the AHG-fluorochrome conjugate), one can use an Fab AHG conjugate which cannot crosslink and thus cannot cause agglutination; such conjugates are available commercially. As mentioned previously, if Fab-AHG is not available, the agglutinated RBCs should be mixed thoroughly with a small bore (0.5-mm) pipette or a 25–27-gauge needle, to disperse most of the agglutination (if the RBCs have been chemically treated to avoid agglutination with primary antibody, the RBCs will also not be agglutinated by the secondary antibody).

In the early 1980s we started applying FC to several areas of blood transfusion medicine, especially red cell immunology (3). The approach has been slow in gaining popularity, but increasing numbers of publications in the 1990s have applied this technology (3,8). Table 1 lists some of the more common applications which are discussed below.

DETECTION AND QUANTITATION OF RBC-BOUND PROTEINS

FC has been used to detect and quantitate RBC-bound IgG (including subclasses), IgM, IgA, and complement (C3).

*Correspondence to: George Garratty, Ph.D., F.R.C.Path., Scientific Director, Southern California Region, American Red Cross Blood Services, 1130 S. Vermont Ave., Los Angeles, CA 90006.
E-mail: garratty@usa.redcross.org
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Table 1
Some Applications of Flow Cytofluorometry to Red Cell Immunology

Detection and quantitation of RBC-bound proteins
Detection of RBC-bound immunoglobulins and complement
Diagnostic aid for autoimmune hemolytic anemia (AIHA)
IgG subclassing
Quantitation of RBC-bound immunoglobulins
Detection and quantitation of RBC antigens and antibodies
Detection and quantitation of minor RBC populations
Blood group mosaicism
Blood group chimerism
Genetic
Bone marrow transplant
Detection and quantitation of transfused RBCs
Phenotyping recipients' RBCs following transfusion
Detection and quantitation of fetal RBCs in maternal blood (FMH)

Table 2
Antiglobulin Test Titration Scores and Flow Cytometric Analysis of RBCs Sensitized In Vitro With Dilutions of Anti-D (32)

Anti-D dilution	Indirect antiglobulin test	Titration score[a]	Mean fluorescence per RBC
1:5	4+	68	212.0
1:10	4+	68	183.2
1:20	4+	62	130.1
1:40	4+	65	84.7

[a]Dilutions of anti-IgG were used to test the anti-D-sensitized RBCs.

Detection of RBC-Bound Immunoglobulins and Complement

As the AGT is so sensitive and is simple and cheap to perform, FC has usually only been applied in special situations. We prepare RBCs for FC analysis in a similar way as for the DAT; RBCs are well-washed with normal saline, and the anti-Ig-conjugate added as soon as possible. Following incubation (30 min at room temperature or 4°C), the RBCs are washed again and analyzed (2).

RBC-bound IgG is detectable by the standard DAT in 1 in 1,400 apparently healthy American blood donors; most of these DATs are only weakly (1+ or less) positive (9–11). If more sensitive assays are used, then RBC-bound IgG (30–40 molecules/RBC) can be shown to be present on the RBCs of most, if not all, individuals (10,11). It is still not clear if IgG is present on all or only some RBCs, whether the amount varies a great deal from cell to cell, and whether it serves a physiologic role. In 1988, Magnani et al. (12) used FC to show that when normal RBCs were separated into fractions according to age, RBC-bound IgG increased as RBCs aged. RBCs from patients with sickle cell disease (SCD) and thalassemia have been found to have increased amounts of RBC-bound IgG (10,11,13–17). Using FC, Green (18) showed that increased amounts of RBC-bound IgA and IgM were also present on the high-density (older) RBC fraction in SCD patients.

RBC-bound complement (C3d and C4d), the C3b/C4b complement receptor (CR1), and the complement control proteins CD55 and CD59, on RBCs, have also been measured using FC (19–26). Patients with paroxysmal nocturnal hemoglobinuria (PNH) lack glycosyl-phosphatidylinositol (GPI)-anchored proteins, including CD55 and CD59, from their subpopulation of affected RBCs. FC analysis, using anti-CD55 and CD59, has been shown to be the best test for diagnosing this rare condition (23–26).

FC as an aid in the diagnosis of AIHA. Some patients have all the clinical and hematological signs of autoimmune hemolytic anemia (AIHA), yet have a negative DAT and no serum antibodies when tested with routine procedures (9,27). Sometimes, small amounts of RBC-bound IgG can be detected by methods more sensitive than the antiglobulin test (9,27). We studied 259 cases of hemolytic anemia of unknown origin, where an immune basis was suspected by the hematologist, but the DAT was reported as negative (27). Five modifications of the DAT, including FC, were used to detect RBC-bound IgG not detected by the standard DAT. FC appeared to be the single best assay (27). FC has been particularly useful for investigating AIHA associated with IgM autoantibodies reacting optimally at 37°C (27–29). RBC-bound IgM can be difficult to detect by the DAT, but is easily detectable by FC where sensitization, not agglutination, is the endpoint. One problem when testing RBCs coated with IgM antibodies is that they often spontaneously agglutinate. When this happens, the agglutinated RBCs can be treated with dithiothreitol or 2-mercaptoethanol to break the disulfide bonds between the pentameric units, leaving IgM monomers on the RBCs. These IgM monomers are not usually detectable by the DAT, but can be detected by FC using fluorochrome-labeled anti-IgM. In some cases, IgM-coated RBCs will show spontaneous agglutination only following centrifugation; this is a problem for the DAT but not for flow cytometric analysis, where a suspension of uncentrifuged RBCs is analyzed.

IgG subclassing. FC has been used to determine the IgG subclass of RBC-bound IgG by using IgG antibodies to human IgG1, IgG2, IgG3 and IgG4, and FITC-anti-IgG conjugates; directly conjugated subclass antisera have also been used (30,31).

Quantitation of RBC-Bound Immunoglobins

Van der Meulen et al. (1) used FC to show that the DAT-positive RBCs of patients with hemolytic anemia appeared to have more RBC-bound IgG than those without hemolytic anemia. They suggested that FC could be used to establish a quantitative threshold for IgG-mediated in vivo RBC destruction. After reading the paper by van der Meulen et al. (1), we set up a simple experiment. We sensitized Rh(D+) RBCs with different amounts of anti-D so that all the RBCs reacted strongly (4+) by the AGT; we modified the routine AGT by testing with serial dilutions of the anti-IgG and expressing the results as a titration score. We also tested the washed anti-D sensitized RBCs with a fluorescein-conjugated anti-IgG using FC. Table 2 shows the results. FC clearly differentiated the RBCs, placing them in order of the expected relative amount of RBC-bound IgG; the AGT did not do this. This impressed us that FC had some major advantages over serology, and stimu-

lated us to apply FC to RBC immunology. It should be mentioned that both direct agglutination and the AGT are remarkably sensitive for routine procedures, detecting about 100–200 IgG molecules per RBC (9–11). Flow cytofluorometry is usually no more sensitive, but in special situations it appears to detect RBC-bound IgG or IgM when the AGT fails (see above). Although the paper by van der Meulen et al. (1) stimulated us to use FC to study RBC interactions, we could not agree with their conclusions, that FC could be used to establish a quantitative hemolytic threshold, when we tried to reproduce their work using a larger number of patients (32). Our results confirmed our belief that the quantity of RBC-bound IgG, although important, is only one of many factors influencing the degree of RBC destruction in a particular patient (10,11,33).

DETECTION AND QUANTITATION OF RBC ANTIGENS AND ANTIBODIES

Antigens

Oien et al. were the first to use FC to study blood group antigens, including Rh (34). Other investigators soon applied the technique to studies of Rh blood group antigens (35–47) (reports up to 1994 were reviewed by Garratty and Arndt (3)). In contrast to indirect methods (i.e., RBCs sensitized with unlabeled Rh antibodies followed by FITC labeled AHG) used by other investigators (34,36,37), von Bockstaele et al. (35), using directly labeled Rh antibodies, could not differentiate weak D (D^u) phenotypes from other D phenotypes. Hasekura et al. (37) used indirect fluorescence to study various Rh phenotypes, with particular reference to very weak D antigens (D^u and D_{el}). Others were also able to distinguish weak D phenotypes from other phenotypes, using indirect fluorescence (39–43). We and others used FC to show a variation in C and c antigen strength (44–46).

Berneman et al. (6), Sharon and Fibach (48), and Fibach and Sharon (49) used FC to study ABH antigens on RBCs. As mentioned previously, this is technically difficult due to the strong agglutination caused by IgM and IgG anti-A and anti-B. Berneman et al. (6) used glutaraldehyde treatment of the RBCs, a method that we have used successfully to overcome agglutination problems. Sharon and Fibach (48) and Fibach and Sharon (49) dispersed agglutination by passing the cells through a 25-gauge needle. Although we have found that we can disperse the more fragile agglutination caused by antiglobulin sera by passing cells through a fine-bore needle, we have not been able to disperse completely agglutination caused by ABO antibodies (unpublished observations, 1993). Fibach and Sharon (49) made the interesting observation that as RBCs age, in vivo, the ABH antigen density decreases on the membrane. They measured this by a two-variable analysis using FITC-conjugated anti-IgG to detect RBC-bound anti-A, -B, and -H on the RBC membrane, and relating this to forward light scatter, which relates to RBC size, which, in turn, relates to the age of the RBC. Popp et al. (50) quantified the loss of B antigenicity, in a patient with acute myeloid leukemia, by flow cytofluorometric analysis. Murai et al. (51) used flow cytofluorometry to quantitate the number of Lewis antigens on RBCs.

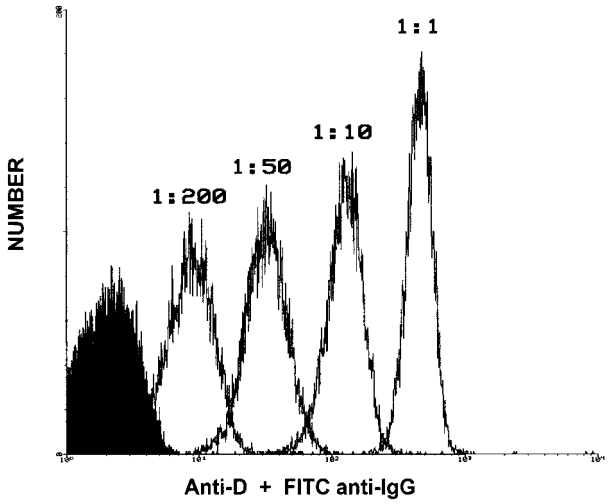

FIG. 1. Composite of five histograms, showing green fluorescence of D+ RBCs after incubation with four dilutions of anti-D (1:1, 1:10, 1:50, 1:200: open histograms) or saline (solid histogram), and then fluorescein isothiocyanate (FITC)-labeled anti-IgG.

FC has been used, with increasing frequency, to study high-frequency blood group antigens, null phenotypes, and RBC membrane chemistry (52–58). Many of the high-frequency antigens have been associated with immunologically important proteins and possible RBC membrane structure and function (59,60). FC has also been used to study the expression of human blood group antigens during erythropoiesis in cell culture systems (61–65).

Oien et al. (66) showed that FC was far superior to the manual titration scores that were commonly used for determining zygosity. When Oien et al. (66) tested RBCs from five heterozygotes and five homozygotes with dilutions of several unselected typing sera, none of the antisera exhibited a clear dosage effect, and there was considerable overlap between titration scores of RBCs from heterozygotes and homozygotes. On the other hand, FC demonstrated a clear dosage effect in 10 of the 20 antisera tested, with clear differentiation of all five homozygotes from the five heterozygotes. FC analysis of RBC blood group antigens of family members has been helpful in paternity testing (67–69), but this is of historical interest only, as now most testing is DNA-based.

Antibodies

If one takes serial dilutions of an antibody (e.g., anti-D) and adds them to antigen positive (D+) RBCs; washes the sensitized RBCs, following incubation; adds FITC; and analyzes the RBCs using FC, the results are as might be expected (see Fig. 1). However, it is more difficult to accurately quantitate antibodies in terms of molecules, micrograms, or international units (IU). In the USA, prenatal titrations of anti-D (using the antiglobulin test) are used to evaluate whether a woman should have invasive tests

such as amniocentesis to assess the status of a fetus possibly affected by hemolytic disease of the newborn. Europeans have, for some years, quantitated antibodies in micrograms or IU, using a standard curve utilizing a standard anti-D and an AutoAnalyzer; more recently, FC has been applied (70–74).

DETECTION AND QUANTITATION OF MINOR RBC POPULATIONS

One of the greatest values of FC to immunohematologists is the efficiency of FC in detecting minor cell populations. The most common applications would be in detecting and quantitating a population of fetal RBCs in the maternal circulation, or detecting and following the number of transfused RBCs, or RBCs from a bone marrow transplant. More unusual applications would be in studying chimeras or RBC mosaicism. In many of the applications, an antigen on the minor RBC population, that is missing from the major RBC population, is selected and targeted with an IgG antibody. The IgG-sensitized minor RBC population is then detected using a fluorochrome anti-human IgG conjugate.

Blood Group Mosaicism

The McLeod phenotype is a rare variant associated with the Kell blood group system (75). In this phenotype, there is very weak expression of Kell antigens on the RBCs. The gene responsible for McLeod (*Xk*), found on the X chromosome, segregates independently from the Kell genes, found on chromosome 7. So far, McLeod phenotypes have only been found in males; the X-linked transmission is through a carrier female. The *Xk* locus is inactivated by the Lyon phenomenon of X chromosome inactivation, and female carriers exhibit cellular mosaicism. Erythropoietic changes include modification of the RBC membrane and acanthocytosis, with reduced RBC survival. Another interesting association of the McLeod phenotype and the rare sex-linked disorder, chronic granulomatous disease (CGD), has been described (75). Thus, identification of the McLeod phenotype, and/or carrier state, may be of great interest to clinicians as well as immunohematologists.

FC has been used for identifying the McLeod carrier state, even when the McLeod cell population was less than 0.1% of the total RBC population (76). We had the opportunity to study the family of a young boy with CGD and the McLeod phenotype (77). As the boy had developed an antibody, anti-KL, that reacted with all RBCs other than those having the McLeod phenotype (only a few donors in the world), we thought that female relatives might be better donors than random donors (i.e., more McLeod-type RBCs than random donors). The propositus showed a depression of k and Kp^b on all his RBCs, and the father showed normal expression of these Kell antigens. The mother and the grandmother showed two populations of cells. One population had depressed Kell antigens and the other had normal expression; 69% of the grandmother's and 60% of the mother's RBCs had depressed Kell antigens (McLeod type) (3,77). Reid et al. (78) reported on the results of an FC study of RBCs from 12 mother-son or father-daughter pairs (6 CGD and 6 non-CGD) with the McLeod phenotype; the proportion of RBCs with depressed Kell antigens ranged from 8–82%.

Blood Group Chimerism

Genetic chimerism. Genetic chimerism (i.e., populations of RBCs from both twins present in the blood of each twin) is extremely rare. We had the opportunity to study a blood donor whose RBCs showed mixed-field agglutination (i.e., small to moderate numbers of agglutinates in a sea of unagglutinated RBCs) with anti-B. FC cleary differentiated a population of B RBCs (35%) and O RBCs (65%). We showed that the two populations were different genetically by testing with anti-Fy^a (64% Fy(a+), 36% Fy(a-)) and anti-Jk^b (63% Jk(b+), 37% Jk(b-)) (3).

Chimerism associated with bone transplantation. RBC antigens have been used to monitor whether the graft has taken following allogeneic bone marrow transplantation (BMT), and to look for disappearance and possible repopulation of autologous RBCs. Serological procedures (i.e., agglutination) were first used to look for minor cell populations with antigenic differences (79,80). The reliable sensitivity of agglutination procedures is felt to be 1 antigen-positive RBC per 100 antigen-negative RBCs, but modified methods can detect down to 1 in 10,000. De Man et al. (81) used fluorescent IgG-coated immunospheres, but did not use a flow cytofluorometer to measure fluorescence. They were able to detect one antigen-positive RBC per 10,000 antigen-negative RBCs, and felt that the method was accurate, sensitive, and reproducible (81).

Blanchard et al. (82) used directly conjugated (FITC) monoclonal and polyclonal blood group antibodies to detect RBCs originating from recipient and donor progenitors. They could detect less than 1% of a minor cell population but felt that 1% or above could be detected unambiguously. Hendricks et al. (83) described an indirect method (i.e., RBCs sensitized with blood group antibody and then tested with conjugated (FITC)-Fab anti-IgG), with a sensitivity of 0.31%. They found a 7.2% intraassay coefficient of variation (CV) for 1% mixtures of D+ RBCs in D– RBCs, and 2.2% for 10% mixtures. Interassay CVs were 9.5% and 6.0%, respectively. Wagner and Flegel (84) also used an indirect method to detect chimerism following bone marrow transplantation. Nelson et al. (85) used an indirect method using unlabeled Rh antibodies to sensitize RBCs followed by a biotin-conjugated sheep AHG and a streptavidin-phycoerythin conjugate. This was followed by treatment with thiazole orange to detect reticulocytes, which would detect the RBCs presently being produced by the bone marrow of the recipient. We have found this approach useful. Figure 2 shows the results of an E– child with β-thalassemia who had received a bone marrow transplant from her E+ sister. The patient's RBCs were incubated with anti-E, phycoerythrin-conjugated anti-IgG, and thiazole orange to determine the percentage of RBCs and reticulocytes that were of donor versus recipient origin. Only 22% of RBCs were E+ (0.5% = reticulocytes) versus 78% which were of the recipient's E– type (5.5%

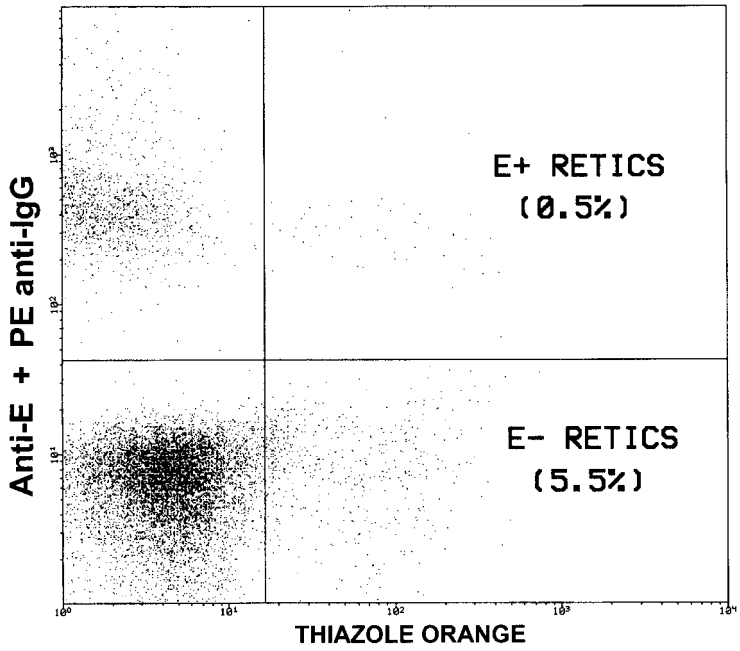

FIG. 2. Two-color dot-plot showing results of testing RBCs from a bone marrow transplant (BMT) patient after incubation with anti-E, phycoerythrin (PE) anti-IgG, and thiazole orange. **Upper left:** BMT donor's E+ mature RBCs. **Upper right:** BMT donor's E+ reticulocytes. **Lower left:** BMT recipient's E− mature RBCs. **Lower right:** BMT recipient's E− reticulocytes.

were reticulocytes). The patient was followed for 2 years, with no change in this picture.

Detection and Quantitation of Transfused RBCs (RBC Survival Studies)

The approach of using FC to detect transfused RBCs in humans usually has been to use an antigen that is present on the transfused RBCs and is not present on the RBCs of the recipient (86–93). Investigators at the New York Blood Center used the opposite approach, of detecting an antigen present on the recipient but absent from the transfused RBCs (76,94–96). It is relatively easy to follow the survival of RBCs when a unit of RBCs has been transfused (e.g., in a delayed hemolytic transfusion reaction), but it is not so easy if one wants to accurately follow the survival of 0.5–1 ml of RBCs, as is used with ^{51}Cr-labeled RBCs.

Postoway et al. (86) reported that FC correlated well with a differential agglutination method for measuring RBC survival in 17 cases of suspected hemolytic transfusion reactions that were investigated. In other reports, the same FC approach was used successfully for RBC survival studies in patients with anti-Jk^a, anti-Js^b, and -Di^b (87,88,91). Garratty et al. (90) used two-color (green and red) fluorescence to differentiate RBCs with acquired B antigen from RBCs with genetically determined B antigen, and to follow the survival of these RBCs in a patient suffering from an ABO hemolytic transfusion reaction.

Valinsky (76) and Valinsky et al. (94) found flow cytofluorometry compared favorably with a ^{51}Cr-labeled RBC survival study of 10 ml of donor RBCs in a patient with AIHA. Issitt et al. (95) compared FC and a ^{51}Cr study in a patient with anti-Lu6. One milliliter of Lu:6 RBCs was labeled with ^{51}Cr and mixed with 9 ml of unlabeled Lu:6 RBCs (to facilitate the flow cytofluorometry study). ^{51}Cr survival at 1 h was 79%, and at 24 h it was 19.2%; survival at 1 h measured by flow cytofluorometry was 85%, and at 24 h was 20%. A similar approach was used to successfully measure the survival of Ge+ RBCs in patients with anti-Ge (96). Lee et al. (92) compared FC with a conventional ^{51}Cr method to study the survival of 4–5 ml RBCs (approximately 0.2% of blood volume); they found that FC was not as sensitive or accurate as the ^{51}Cr method for following survival of ≤5 ml RBCs. In contrast, Perera et al. (93) found that FC performed as well as ^{51}Cr in estimating the survival of 5 ml of Rh(D)-sensitized RBCs. Unfortunately, these two reports (92,93) are in abstract form only, so it is difficult to evaluate the results of each group.

Phenotyping Recipients' RBCs Following Transfusion

It is difficult to ascertain the true phenotype of patients when transfused RBCs are present. Most approaches have depended on separating the younger RBCs, presumed to be the recipient's RBCs, from the older RBCs, presumed to be the transfused RBCs. These methods have usually depended on using substances, such as phthlate esters or centrifugation, to separate young (patient's own) and older (transfused) RBCs because of their size/specific gravity differences. These approaches are not very reliable. Griffin et al. (97) described an FC assay using dual-color flow cytofluorometry. Antigens were labeled with an indirect staining technique, using reagent antisera as the primary antibody and biotinylated goat antihuman IgG as the secondary label, with avidin-phycoerythrin as the

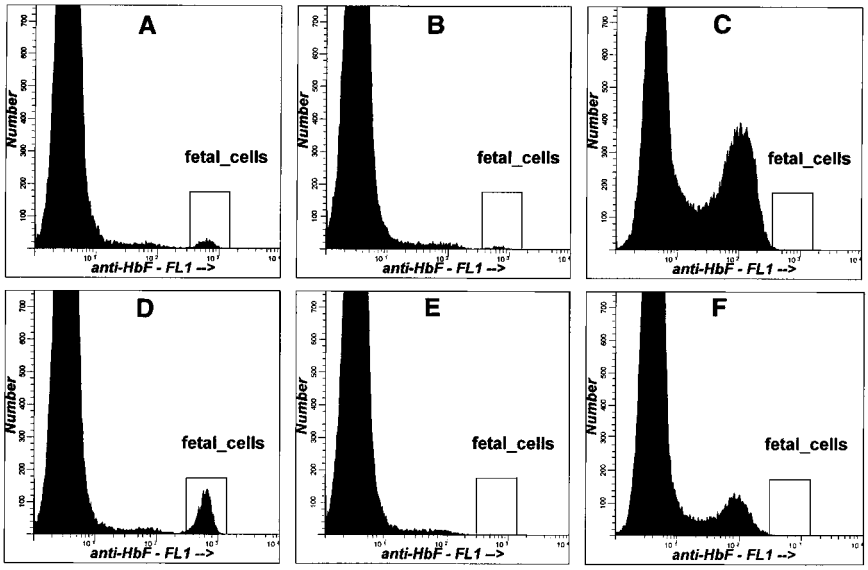

FIG. 3. Common patterns of flow cytometric analysis of blood samples, using monoclonal anti-HbF. The positive control samples of 1% (**A**) and 5% (**D**) fetal cells are used to set the fetal cell region. Typical maternal blood samples, without an increase in F cells, of 0.24% (**B**) and 0.02% (**E**) fetal cells are shown. Specimens with an increase in F cells are seen with hereditary persistence of HbF (**C**) and sickle cell disease (**F**), yielding results of <0.09% fetal cells. Reprinted with permission from Davis BH et al. (114).

fluorescent stain. Reticulocytes were labeled with a thiazole orange fluorescent stain. Thus, the recipients' reticulocytes having the putative antigen would be stained by both fluorochromes, and the older transfused cells by neither, if antigen-negative, or only the avidin-phycoerythrin, if antigen-positive. In vitro simulated transfusion mixtures were tested successfully with anti-c, -C, -K, -s, $-Fy^a$, $-Fy^b$, $-Jk^a$,and $-Jk^b$. The in vitro results were confirmed in 19 transfused patients who received blood with known antigens foreign to their own (97). Others (85), including our laboratory (unpublished data), have used this approach successfully. Wagner (98) recently used two-color indirect fluorescence to phenotype (c, Fy^a, Jk^a) the minor population of D+ RBCs in a patient transfused with D- RBCs.

Detection and Quantitation of Fetal RBCs in Maternal Blood (Fetal-Maternal Hemorrhage; FMH)

In the USA, all Rh(D)-negative women receive prophylactic RhIg at 28 weeks of pregnancy and again within 72 h of delivery, to prevent Rh alloimmunization from fetal D+ RBCs. One dose of RhIg is enough to deal with an FMH of 15 ml (approximately 0.6%) fetal RBCs. Maternal blood is tested for the presence of fetal RBCs. If fetal RBCs are detected, the amount has to be quantitated to enable the appropriate dose of RhIg to be administered.

The quantitative assay for FMH generally used is the Kleihauer-Betke (K-B) acid elution method (99), which relies on the difference between adult and fetal hemoglobin's resistance to acid elution. The method has been shown to be subjective and to have poor reproducibility (100–102). In a survey of four hospitals, Warzynski (102) found a coefficient of variation (CV) of 150% for a 0.33% FMH. More sophisticated methods, such as the enzyme-linked antiglobulin test and FC, are more objective, sensitive, and reproducible than the standard methods but tend to be more complex and time-consuming, and to require special apparatus such as a spectrophotometer or flow cytofluorometer. Nevertheless, flow cytofluorometers are becoming more available, and recent approaches (103–115) using FC are much less complex than those previously described (116,117).

Sensitive methods such as those described by Medearis et al. (116) and Cupp et al. (117) might have applications in specialized projects where such sensitivity is important, but for the routine evaluation of FMH, with regard to RhIg dosage, the sensitivity needed is only about 1 fetal RBC in 200 maternal RBCs (i.e., 0.5% FMH).

Nance et al. (103,104) described a relatively simple indirect FC method to detect FMH. RBCs from the D- mother were incubated with IgG anti-D. After incubation, FITC anti-IgG was added to the washed RBCs. The sensitivity of this method, with acceptable reproducibility, was 1 fetal (D+) RBC per 1,000 maternal (D-) RBCs. The method was found to be precise and reproducible for quantitating fetal cells in the 0.5% critical range necessary for evaluating the need for extra doses of RhIg. Other workers have since used a similar indirect approach and came to the same conclusions (105–107,109). Direct approaches using fluorochrome-labeled anti-D have been used (108,110–112). Nelson et al. (108) used a direct method utilizing biotinylated monoclonal anti-D, followed by a fluorescent-conjugated streptavidin. They claimed that this test was sensitive, reproducible, cheap, and fast. The report by Bayliss et al. (107) is of particular interest, as they compared five different methods, including FC. Nance et al (36) showed that their technique could also detect fetal RBCs with weak D antigens (D^u) in a D- mother, and fetal

D+ RBCs in a mother with a ''partial D'' (category VI) phenotype. The detection of D+ RBCs in a population of RBCs with a weak D phenotype is not possible using the ''microscopic D^u'' or rosette procedures for FMH. Lloyd-Evans et al. (118) recently suggested that the use of a PE-conjugated anti-glycophroin-A as a double label improves the accuracy of FMH by FC.

The most recent approach for detecting FMH does not depend on the presence of D antigen on the fetal RBCs, but rather the presence of fetal hemoglobin (HbF) (114,115). Davis et al. (114) and Nelson et al. (115) used a monoclonal antibody to HbF conjugated to FITC. The RBCs have to be permeabilized using glutaraldehyde and Triton X-100 to allow access to the intracellular HbF. The method correlated well with the acid elution method and had superior precision (CV of $<15\%$) with $>0.1\%$ fetal RBCs (114). Hereditary persistence of HbF could be differentiated from fetal RBCs in the maternal blood (Fig. 3) (114).

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